Occurrence of antibiotic-resistant bacteria and endotoxin associated with the land application of biosolids

J.P. Brooks, S.L. Maxwell, C. Rensing, C.P. Gerba, and I.L. Pepper

Abstract: The purpose of this study was to determine the prevalence of antibiotic-resistant bacteria and endotoxin in soil after land application of biosolids. Soil was collected over a 15 month period following land application of biosolids, and antibiotic resistance was ascertained using clinically relevant antibiotic concentrations. Ampicillin, cephalothin, ciprofloxacin, and tetracycline resistance were all monitored separately for any changes throughout the 15 month period. Endotoxin soil concentrations were monitored using commercially available endotoxin analysis reagents. Overall, land application of biosolids did not increase the percentage of antibiotic-resistant culturable bacteria above background soil levels. Likewise, land application of biosolids did not significantly increase the concentration of endotoxin in soil. This study determined and established a baseline understanding of the overall effect that land application of biosolids had on the land-applied field with respect to antibiotic-resistant bacterial and endotoxin soil densities.

Key words: biosolids, antibiotic resistance, endotoxin, groundwater, land application.

Résumé : Le but de cette étude était d'examiner la prévalence de bactéries résistantes aux antibiotiques et déterminer la présence d'endotoxines après l'application de solides biologiques à la surface du sol. Le sol a été récolté au cours d'une période de 15 mois après l'application des solides biologiques et la résistance aux antibiotiques a été évaluée dans une gamme de concentrations d'antibiotiques pertinentes d'un point de vue clinique. Les changements de résistance à l'ampicilline, à la céphalothine, au ciprofloxacin et à la tétraclycine ont été évalués séparément pendant toute la période de 15 mois. Les concentrations d'endotoxines du sol ont été mesurées à l'aide de réactifs d'analyse d'endotoxines commerciaux. Globalement, l'application de solides biologiques à la surface des sols n'a pas augmenté le pourcentage de bactéries cultivables résistantes aux antibiotiques par rapport à la ligne de base de sols contrôles. De la même façon, l'application de solides biologiques à la surface des sols n'a pas augmenté significativement la concentration d'endotoxines du sol. Cette étude a permes de définir une connaissance de base des effets globaux que l'application de solides biologiques peut avoir sur les champs traités en regard de la densité de bactéries résistantes aux antibiotiques et de la présence d'endotoxines dans les sols.

Mots-clés : solides biologiques, résistance aux antibiotiques, endotoxines, eau souterraine, application sur des sols.

[Traduit par la Rédaction]

Introduction

Activated sludge sewage treatment results in the production of large amounts of biosolids, which are typically disposed of or recycled through land application to agricultural land. In the United States, more than half of all biosolids are applied to farmland, and with that comes concern about the potential health and environmental effects of antibiotic-resistant bacteria and endotoxins in land-applied biosolids (National Research Council 2002). The majority of these biosolids are Class B biosolids. Class B biosolids are produced when sewage sludge has been chemically or physically treated to reach an acceptable fecal coliform level of at most 2×10^6 most probable number (MPN)·(g total solid mass)$^{-1}$ (National Research Council 2002). Class B biosolids are known to contain some pathogenic microorganisms, antibiotic-resistant microorganisms, and bacterial by-products such as lipopolysaccharide.

The antibiotic era began after Alexander Fleming's discovery of penicillin nearly 80 years ago. Since the first introduction of antibiotics, overuse has been an issue, as overprescription of first generation antibiotics has led to many resistant bacterial strains (Murray et al. 1998; Monroe and Polk 2000; Lieberman 2003). The presence of antibiotic-resistant bacteria in wastewater has been investigated and thought to be more related to hospital rather than domestic waste (Valdivia et al. 1996); however with regard to biosolids, little research has been conducted. As such, biosolids may be highly influenced by hospital waste and any associated antibiotic-resistant bacterial populations. Human

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J.P. Brooks.[1] Department of Soil Water and Environmental Science, University of Arizona, Tucson, AZ 85721, USA; United States Department of Agriculture, Agriculture Research Service, Waste Management and Forage Research Unit, 810 Highway 12 East, Mississippi State, MS 39762, USA.
S.L. Maxwell, C. Rensing, C.P. Gerba, and I.L. Pepper. Department of Soil Water and Environmental Science, University of Arizona, Tucson, AZ 85721, USA.

[1]Corresponding author (e-mail: jbrooks@msa-msstate.ars.usda.gov).

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bacterial pathogens, such as *Salmonella*, *Shigella*, and *Campylobacter*, can all be potentially present in biosolids, and as such may present a cause for concern when antibiotic resistance is involved. In addition to these pathogens, biosolids may harbor additional antibiotic-resistant pathogenic and nonpathogenic microorganisms (Rusin and Gerba 2001). Antibiotic resistance is typically due to the intrinsic resistance inherent to many of these organisms, to resistance selection over time, or to potential horizontal gene transfer, which could include antibiotic resistance (Low 2001; Rusin and Gerba 2001; Rensing et al. 2002; Dzidic and Bedekovic 2003; Marshall et al. 2004; Salyers et al. 2004). Therefore, potentially when soil, water, or food that has been in contact with biosolids is consumed either directly or indirectly, there exists the possibility of exposure to these antibiotic-resistant bacterial strains.

Endotoxin, or the lipopolysaccaride (LPS) molecules associated with the Gram-negative bacterial outer wall, are molecules capable of soliciting large-scale immune reactions when introduced into a susceptible individual. The prevalence of endotoxin in biosolids has not been well studied, although it is assumed that biosolids can potentially contain large amounts of endotoxin because of its high concentration of Gram-negative bacteria (Raetz and Whitfield 2002). Environmental health effects associated with the endotoxin group of molecules is primarily associated with inhalation complications, rather than with consumption; however, little information exists to suggest that consumption is not a concern (Castellan et al. 1987; Smid et al. 1992; Donham et al. 2000; Gereda et al. 2001; Michel et al. 2001; Michel 2003). Very little is known on the overall prevalence of endotoxin following its introduction into the environment; however, it is widely accepted that lipopolysaccaride molecules are ubiquitously present in the environment, and as such they may or may not be influenced greatly by the addition of foreign LPS.

The purpose of this study was to quantify the overall amount of antibiotic-resistant bacteria and endotoxin present in biosolids and in the soils that received biosolids. The primary focus of this study was to determine soil concentrations of these contaminants during pre- and post-biosolids land application periods on an experimental agricultural field. This study established a baseline set of data related to biosolids with respect to potential environmental contamination with antibiotic-resistant microorganisms and endotoxin.

Materials and methods

Experimental site and biosolids application

An agricultural site within the Tucson, Arizona, area was monitored throughout 15 months following the land application of Class B biosolids. Liquid Class B biosolids from the Ina Road Wastewater Treatment Plant located in Tucson, Arizona, were applied from a Balzer 6250 gallon capacity injector applicator (Balzer Inc; Mountain Lake, Minnesota) with injection occurring approximately 15 cm below the sandy–loam soil surface. Biosolids were applied at a rate of 5452 kg (dry)·ha^{-1}. All biosolids were anaerobically digested and were approximately 6%–8% solid content. Class B biosolids had previously been applied to the experimental site in December of 1995, with the field subsequently utilized for the growth of cotton.

In addition to the experimental field, a set of 5 local agricultural fields, which had no record of biosolids application, were visited as nonapplied control sites. An off-site agricultural field, which annually received anaerobically digested Class B biosolids during the past 20 years, was also visited. All sampled sites were characterized as having sandy–loam soil with approximately 6% moisture content.

Sample collection

Soil samples were collected prior to and following biosolids land application at specific time points: Day 1 (pre-application); Days 0, 7, and 14; Months 1, 2, 3, 4, 5, 6, and 15. Land application at the experimental site began on 10 June 2003 (Day 0). Soil samples were also collected from the off-site nonapplied control sites and the continuously applied control site. Composite soil samples were collected from all sites at approximately 15 cm below the surface, using a disinfected (70% ethanol) sampling shovel. All soil samples were sieved through a 2 mm pore size sieve. Soil moisture content measurements were made prior to analysis.

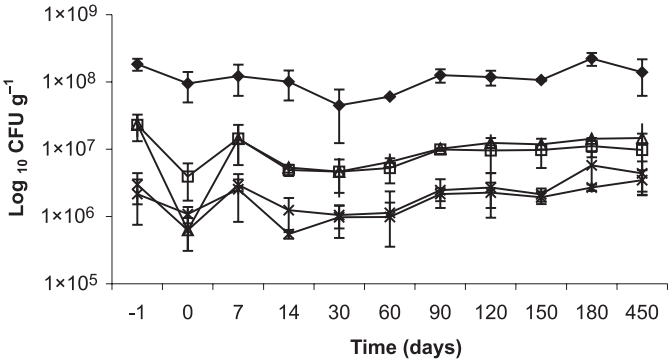
In addition to the soil samples, Class B anaerobically digested biosolids samples were also collected. A composite of the anaerobically digested Class B biosolids, which were applied to the experimental field, was collected to ascertain pre-application levels of biosolids-borne antibiotic-resistant bacteria. A set of 4 randomly selected biosolids samples from the Arizona, California, New Hampshire, and Washington states was also collected to determine antibiotic-resistant bacterial concentrations within Class B anaerobically digested biosolids from other regions of the country. All samples were stored in an ice cooler and transported back to the laboratory for immediate sample processing. Biosolids solid content analysis was performed prior to microbial analysis. Soil and biosolids aliquots to be analyzed for the presence of endotoxin were frozen at –20 °C prior to analysis.

Antibiotic-resistant bacterial analysis

To determine antibiotic-resistant bacterial (ARB) concentrations, samples were exposed to 4 separate antibiotics via dilution and plating on media amended with antibiotics. The 4 antibiotics (Sigma Aldrich; St. Louis, Missouri) chosen were ampicillin (32 μg·mL^{-1}), cephalothin (32 μg·mL^{-1}), ciprofloxacin (4 μg·mL^{-1}), and tetracycline (16 μg·mL^{-1}). Each antibiotic represents a major class of antibiotic and susceptibility range: ampicillin (Penicillin class, broad spectrum), cephalothin (Cephalosporin class, narrow spectrum), ciprofloxacin (Quinolone class, broad spectrum), and tetracycline (Tetracycline class, broad spectrum). In addition, each one represents a specific method of activity, such as peptidoglycan layer formation inhibition (ampicillin, cephalothin), DNA gyrase inhibition (ciprofloxacin), and protein formation inhibition (tetracycline).

Plating medium (R2A; Becton Dickinson, Sparks, Maryland) was amended with these clinically relevant antibiotic concentrations (Jorgensen et al. 1999). Each antibiotic was individually amended into the R2A medium containing an additional antifungal cyclohexamide (Sigma Aldrich) (200 μg·mL^{-1}) additive. Soil samples were first suspended in sterile distilled water and subsequently serially diluted

Fig. 1. Heterotrophic plate count and antibiotic-resistant bacterial concentrations (vertical bars indicate standard deviation) from an experimental field land applied with Class B biosolids and monitored for 15 months. Antibiotics used were as follows: □, ampicillin (32 μg·mL^{-1}); △, cephalothin (32 μg·mL^{-1}); x, ciprofloxacin (4 μg·mL^{-1}); and *, tetracycline (16 μg·mL^{-1}). ♦, Heterotrophic plate count. Day –1 refers to before-application time periods and Day 0 refers to day of application.



prior to spread plating 0.1 mL of each dilution onto each antibiotic-amended plate. Plates were incubated for 5 days at 27 °C. Heterotrophic plate count (HPC) bacterial concentrations were determined by plating onto R2A agar containing only the cyclohexamide additive. Antibiotic-resistant percentages were derived by comparison of heterotrophic culturable concentrations and antibiotic-resistant culturable concentrations. All assays were performed in duplicate.

Endotoxin analysis

Samples were assayed via the use of the commercially available *Limulus* amebocyte lysate assay (Sigma-Aldrich). In the presence of endotoxin, *Limulus* amebocyte lysate forms a gel, confirming the presence of both bound and unbound endotoxin. All endotoxin assays were performed under depyrogenated conditions. Specifically, glass culture tubes, dilution water, and pipette tips were all depyrogenated either commercially or onsite. Glass culture tubes were depyrogenated by baking the glassware at 180 °C for 3 h.

Prior to analysis, frozen biosolids and soil sample aliquots were thawed in a room temperature water bath, followed by suspension in depyrogenated water (Abott Laboratories, Chicago, Illinois) at a concentration of 5 mg·mL^{-1}. Samples were vortexed for 20 min at high speed and appropriate serial dilutions were made.

A 0.1 mL sample aliquot and 0.1 mL of *Limulus* reagent were incubated together following vigorous vortexing for 30 s. All samples were assayed in duplicate. In addition to all samples, control tubes containing diluted known amounts of purified endotoxin (Sigma-Aldrich), ranging from 1.0 to 0.03 endotoxin units (EU)·mL^{-1}, were prepared in depyrogenated water and assayed to ascertain assay sensitivity. In addition to these controls, sample inhibition, positive, and negative controls were all prepared and assayed in duplicate. Inhibition controls were prepared using sample preparations (soil or biosolids in depyrogenated water) that had been processed for endotoxin and spiked with a known amount of endotoxin equivalent to the positive control or 0.3 EU·mL^{-1}. Sample inhibition controls were required to perform as well as the positive controls. Any failed inhibition controls resulted in sample dilution to remove any inhibition. Positive controls were prepared by mixing endotoxin to a concentration of 0.3 EU·mL^{-1} in depyrogenated water, while negative controls were prepared by using depryogenated water. All samples were incubated at 37 °C in a static water bath immediately following reagent addition and mixing. Following the 1 h incubation, samples demonstrating the formation of a solid gel-like phase were deemed positive. To determine a positive gel phase, samples were carefully inverted once, and a positive sample was noted by the presence of a solid gel phase, which remained in the tube. The concentration of endotoxin present in the sample was determined by using the reciprocal of the final dilution presenting a positive result (i.e., duplicate tubes must form gel phase) and multiplying it by the lowest determined control standard concentration.

Statistics

Statistical analyses were performed using the Minitab statistical program version 13.32 (Mintab Inc; State College, Pennsylvania).

Results

HPC and ARB concentrations — land application site

Total culturable HPC bacterial concentrations throughout the study period were monitored at the land application site. Overall, HPC soil concentrations did not deviate from the pre-application concentrations ($P > 0.05$) (Fig. 1). Total HPC concentrations averaged approximately 10^8 CFU·g^{-1} prior to biosolids application and remained similar throughout the 15 months following application (Fig. 1). HPC concentrations from the nonapplied control fields varied from 9.60×10^7 to 1.33×10^8 CFU·g^{-1} (Fig. 2). These levels were found to be statistically similar to that of the experimentally applied field. HPC concentrations from the continuously applied field were approximately 2.55×10^8 CFU·g^{-1}.

ARB soil concentrations in the monitored site were also relatively constant throughout the study period (Fig. 1). Likewise, antibiotic-resistant rates among total bacterial concentrations did not differ throughout the entire study period (Table 1). Ampicillin-, cephalothin-, ciprofloxacin-, and tetracycline-resistant bacterial concentrations in the biosolids-applied field did not statistically differ ($P > 0.05$) throughout the study period, in fashion similar to that of the HPC soil concentrations.

Soil samples collected from the nonapplied control sites contained varying concentrations of antibiotic-resistant bacteria ranging from 2.53×10^6 to 1.06×10^7 CFU·g^{-1} (Fig. 2). Statistical analysis revealed no difference between ARB concentrations from the nonapplied control sites and the experimentally applied site. Likewise antibiotic-resistant rates were found to be statistically similar (Table 2). Samples collected from the continuously applied field were found to contain ARB concentrations from 7.9×10^6 to 3.08×10^7 CFU·g^{-1}.

In addition to field measurements, a composite sample of the anaerobically digested Class B biosolids, which were applied to the field, were analyzed for the presence of biosolids origin antibiotic-resistant and HPC bacteria (Fig. 2).

Fig. 2. Heterotrophic plate count (HPC) and antibiotic-resistant bacterial concentrations (vertical bars indicate standard deviation) detected in Class B biosolids (Biosolids) collected from other regions of the USA, in Class B biosolids used in the experimental application field (Biosolids-App), nonapplied field controls (Field Non-App), and in biosolids from continuously applied (Cont. App.) sites. Antibiotics used were as follows: Amp, ampicillin (32 μg·mL^{-1}); Cep, cephalothin (32 μg·mL^{-1}); Cipro, ciprofloxacin (4 μg·mL^{-1}); and Tet, tetracycline (16 μg·mL^{-1}). Biosolids-App and Cont. App. used in this study were represented by only one composite sample each.

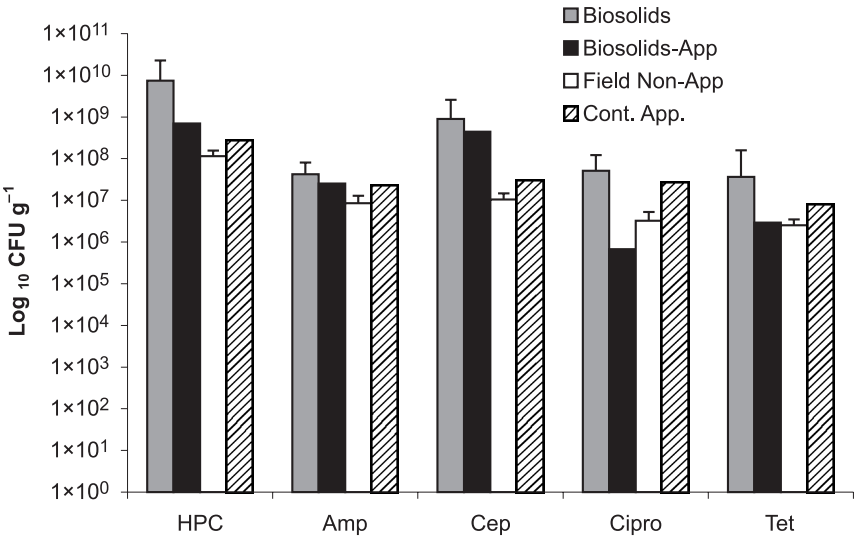


Table 1. Percentage of total culturable heterotrophic plate count (HPC) bacterial concentrations exhibiting antibiotic resistance from soil collected at the experimental land application site throughout the 15 month study period.

	Field antibiotic resistance (%)			
Time	Ampicillin	Cephalothin	Ciprofloxacin	Tetracycline
–1	12.1	12.4	1.6	1.1
0	5.3	7.9	0.9	1.4
7	11.5	11.5	2.8	2.0
14	5.4	6.1	1.5	0.6
30	14.6	13.6	4.8	3.1
60	8.7	10.7	1.9	1.6
90	8.2	8.4	2.1	1.8
120	8.4	11.0	2.2	2.0
150	9.1	11.1	2.0	1.8
180	5.3	6.6	2.7	1.3
450	6.7	10.5	3.3	2.7

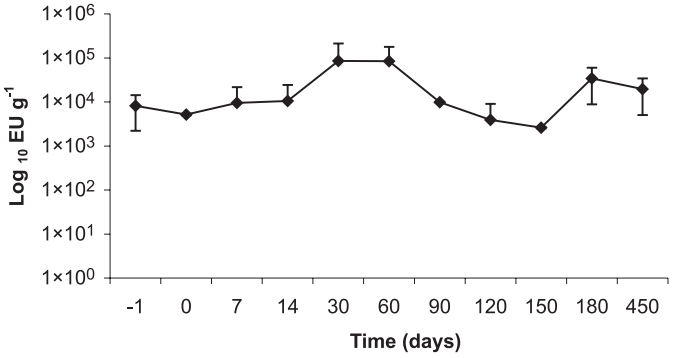
Note: Field antibiotic resistance percentage was calculated by dividing the antibiotic-resistant bacterial concentration by the HPC bacterial concentration. Antibiotics and the concentrations used are as follows: ampicillin (32 μg·mL^{-1}), cephalothin (32 μg·mL^{-1}), ciprofloxacin (4 μg·mL^{-1}), and tetracycline (16 μg·mL^{-1}).

HPC bacterial concentrations were approximately 7.02 × 10^8 CFU·g^{-1}, while ARB concentrations varied from 6.78 × 10^5 to 4.46 × 10^8 CFU·g^{-1}. Similarly, ARB and HPC concentrations from other regional Class B biosolid samples were found to contain similar bacterial concentrations in all regards (Fig. 2).

Endotoxin concentration — land application site

Overall, endotoxin concentrations demonstrated similar trends to that of the ARB concentrations (Fig. 3). Following land application of biosolids, endotoxin concentrations from the applied soil did not differ significantly from pre-application concentrations ($P > 0.05$). Concentrations did increase by approximately 0.5 $\log_{10}$ 1 month after application; however, levels were not statistically significant when compared with pre-application levels. Likewise, endotoxin concentrations from the nonapplied control fields were 7.96 × 10^3 EU·g^{-1}, which were not statistically different from that of the experimentally applied field (Fig. 4). Endotoxin concentrations from the continuously applied field were determined to be upwards of 2.11 × 10^4 EU·g^{-1}. These levels were approximately one-half a $\log_{10}$ greater than that of the nonapplied control sites.

Fig. 3. Soil endotoxin concentrations (vertical lines indicate standard deviation) detected from an experimental field applied with biosolids.



A composite sample of Class B biosolids, prior to land application, was assayed to determine levels of endotoxin present in the biosolids. This sample yielded pre-application biosolids endotoxin concentrations at approximately 8.83 × 10^6 EU·g^{-1} (Fig. 4). Likewise, endotoxin concentrations from the randomly selected Class B biosolids samples collected from 3 of the 4 US states were determined to be approximately 6.12 × 10^5 EU·g^{-1}, while one sample was found to be approximately 5.71 × 10^9 EU·g^{-1} (Fig. 4).

Discussion

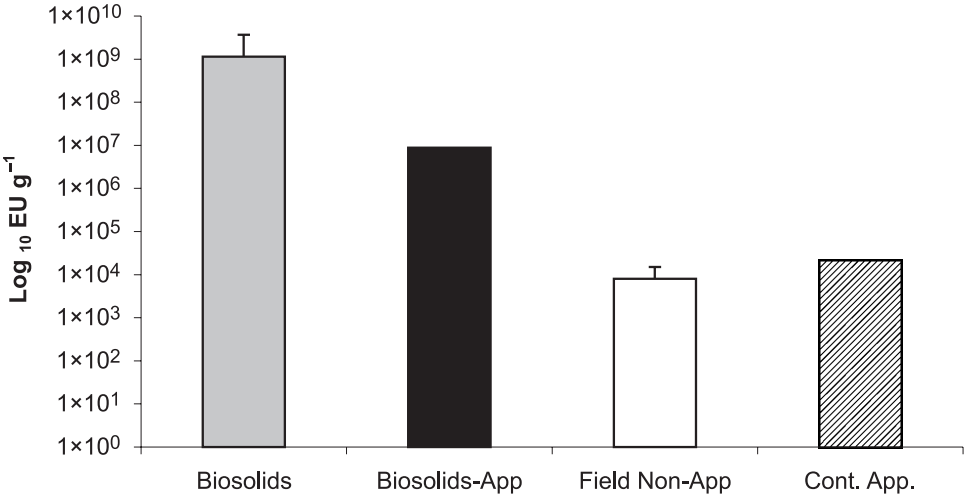
Both HPC and ARB concentrations in soil collected

Table 2. Percentage of total culturable heterotrophic plate counts (HPC) bacterial concentrations exhibiting antibiotic resistance in Class B biosolids (Biosolids) collected from other regions of the USA, in Class B biosolids used in the experimental application field (Biosolids-App), in nonapplied field controls (Field Non-App), and in biosolids from continuously applied (Cont. App.) sites.

	Antibiotic resistance (%)			
	Ampicillin	Cephalothin	Ciprofloxacin	Tetracycline
Biosolids	4.4	21.2	1.8	1.9
Biosolids-App	3.6	63.6	0.1	0.4
Field Non-App	8.1	10.1	3.1	2.4
Cont. App.	7.9	11.0	9.2	2.8

Note: Field antibiotic resistance percentage was calculated by dividing the antibiotic-resistant bacterial concentration by the HPC bacterial concentration. Antibiotics and the concentrations used are as follows:ampicillin (32 $\mu g \cdot mL^{-1}$), cephalothin (32 $\mu g \cdot mL^{-1}$), ciprofloxacin (4 $\mu g \cdot mL^{-1}$), tetracycline (16 $\mu g \cdot mL^{-1}$).

Fig. 4. Endotoxin concentrations (vertical lines indicate standard deviation) detected in Class B biosolids (Biosolids) collected from other regions of the USA, in Class B biosolids used in the experimental application field (Biosolids-App), in nonapplied field controls (Field Non-App), and in biosolids from continuously applied (Cont. App.) sites. Biosolids-App, and Cont. App. used in this study were represented by only one composite sample each.



from the application site remained statistically similar to pre-application concentrations throughout the study. Unexpectedly, the soil concentrations (post-application) remained consistent with and even decreased below pre-application levels with regard to ARB concentrations. Any numerical anomalies noted throughout the study period for both ampicillin- and cephalothin-resistant concentrations may have been due to either a deviation in soil sampling precision or cyclical differences in field bacterial population dominance (Smit et al. 2001). Ciprofloxacin- and tetracycline-resistant bacterial concentrations remained constant throughout the study period, in a fashion similar to that of ampicillin- and cephalothin-resistant concentrations. It is possible that following biosolids land application, the lack of selective pressures for antibiotic resistance may cause the loss of the plasmid carrying the resistant genes and, hence, may lead to lower overall antibiotic-resistant bacterial recovery (Smith and Bidochka 1998). Furthermore, the overall loss of bacterial viability following biosolids land application may have contributed to any noted decreases (Pepper et al. 1993; Zaleski et al. 2005); however, all differences were statistically irrelevant. No increase in ARB concentrations or percentages was noted throughout the study period, particularly, during the first 14 days following application, suggesting that an immediate dilution effect of any biosolids-borne antibiotic-resistant organisms may be the cause. This was true, despite some ARB concentrations in the biosolids to be approximately 1 $\log_{10}$ above that of the pre-application soil concentrations. Soil ARB rates were well below 20% for all measured antibiotics before and after biosolids application. In contrast, dairy soil, farm water, and surface water have all been found to contain ARB rates in upwards of 70% of the culturable total bacteria when constantly exposed to the contamination source (Esiobu et al. 2002). Of course this effect may be more pronounced because of more constant selective pressures on these environments, whereas in the present study, a one-time application of biosolids was the only noted selective pressure. Interestingly, a continuously biosolids applied field within the sampling area was also investigated for this study and found to contain similar ARB concentrations to that of the experimental field, despite Class B biosolids application occurring on an annual basis for the past 20 years. It is important to note, that only one continuously

applied field was investigated for this study, and thus, no statistical analysis was conducted using this particular field, therefore, these numbers hold no statistical relevance. However, 5 other nonbiosolids applied fields were also investigated and found to be statistically similar to the experimental field, with regard to ARB concentrations.

Endotoxin concentrations in the monitored field changed little following biosolids land application. The biosolids used in this study were found to contain endotoxin concentrations similar to that of other Class B biosolids sampled from other regions of the USA. These concentrations were approximately 2 $\log_{10}$ orders of magnitude greater than the pre-application soil levels and were noted to have not affected the soil endotoxin levels following biosolids application. A 0.5 $\log_{10}$ increase in endotoxin concentration 1 month following biosolids land application was noted; however, this level was found to be statistically identical to pre-application levels. This small increase may have been due to Gram-negative bacterial decay in the field, since Class B biosolids contain approximately 10^6 CFU total coliforms·(g dry mass)$^{-1}$, not to mention the other dominant Gram-negative microorganisms, and each of these cells can potentially contain approximately 10^6 lipid A residues (Raetz and Whitfield 2002). Endotoxin, of which lipid A is a key component, can be liberated during Gram-negative decay as well as growth (Bradley 1979), and as such, an increase in overall endotoxin concentration was expected.

Endotoxin generally causes ailments after inhalation of airborne endotoxin, and as such, any potential increase of soil endotoxin concentrations could then lead to increases in potentially aerosolized endotoxin (Brooks et al. 2006). Data from this study suggest that pre-application endotoxin levels in soil were statistically similar to those of post-application levels, and as such, it is likely that aerosols generated by land application operations are likely to contain endotoxin regardless of the presence or absence of biosolids, as demonstrated by a recent study (Brooks et al. 2006).

Overall biosolids did little to alter the overall concentrations of antibiotic-resistant bacteria in a biosolids land-applied field, which was monitored for 15 months, despite expectations of the contrary. It is important to note that this study demonstrated overall antibiotic resistance to only one antibiotic at a time and to only 4 antibiotics. Clinically relevant concentrations of each antibiotic were studied; however, further work must be done to characterize specific isolates with regard to specific resistance and the presence of multiple antibiotic resistance. It is important to note that even resistance to one antibiotic can demonstrate the presence of resistance to other antibiotics, and though 4 antibiotics were investigated, more research is warranted on other antibiotics. This study exhibited that the overall intrinsic levels of antibiotic- (ampicillin, cephalothin, ciprofloxacin, and tetracycline) resistant bacteria already present in the field were unaffected by the land application of Class B biosolids. However, this study did not investigate dominant field ARB isolates, which may have been shifted due to the land application event, and as such, this point should not be overlooked in future studies. It is also important to note that this study does not represent the total viable bacterial community, and that all antibiotic-resistant viable but not culturable bacteria were not represented in this study.

This study can be used as a baseline understanding of the overall amounts of antibiotic-resistant bacteria and lipopolysacharide (endotoxin) that contributed to a single experimental field following biosolids application. Class B biosolids land application did little to alter the intrinsic concentrations of either antibiotic-resistant bacteria or lipopolysacharide present in the soil. However, it is important to understand that biosolids from only one municipality was used and only one experimental field was investigated. Research in other environments is needed, as specific climate and soil characteristics will alter some results. While this study determined quantitative effects of biosolids land application, it is important to note that further research must be conducted to ascertain any qualitative characteristic shifts, such as changes in microbial population, that can lead to shifts in ARB populations and lipopolysacharide (endotoxin) types.

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